# Response of Carcinogen-Altered Mouse Epidermal Cells to Phorbol Ester Tumor Promoters and Calcium

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Primary cultures of mouse epidermal cells are induced to terminally differentiate when extracellular calcium levels are increased to more than 0.1 mM. After carcinogen treatment, cellular foci can be selected that resist this calcium signal to terminally differentiate. Calcium causes these foci to stratify; however, in contrast to normal epidermis, DNA-synthesizing cells in these foci are found in the suprabasal cell layers as well as in basal cells. Cell lines derived from these foci may be considered to be putative initiated cells. Three of these cell lines, designated 308, D, and F, have been characterized for their response to calcium and phorbol ester tumor promoters. The formation of cornified cells and the activity of epidermal transglutaminase were utilized as markers of epidermal differentiation. Neither calcium nor the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) increased transglutaminase activity or cornification of any of the 3 lines. Proliferation was estimated by the [$^3$H]thymidine labeling index, by incorporation of [$^3$H]thymidine into DNA, and by a clonal growth assay. Unlike primary normal cultures, raising the calcium level of the medium did not markedly reduce the rate of proliferation of any of the 3 cell lines. In 2 of the lines, line 308 and line D, proliferation increased in response to TPA exposure. In line F, [$^3$H]thymidine incorporation in confluent cultures was inhibited by TPA, while in cells plated at clonal densities, TPA was cytotoxic at doses of 5 ng/ml or higher. If these calcium-resistant epidermal cell lines correspond to initiated cells, their lack of sensitivity to the induction of terminal differentiation by TPA could account for their growth relative to normal cells. Those lines that also respond to stimulation of proliferation by TPA to a greater extent than normal cells would have a further growth advantage. *J Invest Dermatol 88:60–65, 1987*

Mouse epidermal cell cultures derived from skin exposed to carcinogens in vivo or primary cultures exposed to carcinogens in vitro yield foci of cells that are resistant to calcium-induced terminal differentiation [1]. Such foci are defined by the ability to grow in medium containing >0.1 mM calcium while normal cells undergo a characteristic pattern of terminal differentiation in such medium [2]. Characteristics of this assay that suggest that it selects for initiated cells include: (1) resistant foci can be isolated from mouse skin that had been initiated in vivo even up to 10 weeks prior to isolation of cells for culture [3,4]; (2) the number of foci increased with increasing doses of initiator in vivo or in vitro [4]; (3) stronger initiators yielded more foci than weaker initiators for both in vivo and in vitro exposures [4,5]; (4) the number of foci induced by benzo[a]pyrene directly correlated with the extent of benzo[a]pyrene binding to DNA after in vitro exposure [6]; (5) spontaneous foci developed in cultures from SENCAR mice which are sensitive to papilloma formation by promotion alone [4]; and (6) foci are not tumorigenic when first formed, but cell lines derived from foci may progress to produce carcinomas upon in vivo testing [7]. These studies suggested that carcinogens can alter the normal program of epidermal differentiation as an early event in transformation, perhaps at the initiation stage. A number of cell lines have been derived from these carcinogen-altered foci and some of their characteristics have been described [7].

Tumor promotion in mouse skin by phorbol esters may result from the selective clonal expansion of initiated cells [8–10]. The selective advantage of initiated cells results from a regenerative hyperplasia induced in mouse skin by phorbol esters which occurs because phorbol esters induce terminal differentiation in a subpopulation of mouse basal keratinocytes [11]. According to this hypothesis, initiated cells are resistant to this differentiative influence of phorbol esters and thus would clonally expand as basal cell space became available as a result of the loss of differentiating normal cells. Parkinson et al [12] found that papilloma cell line MEK 79 was resistant to the inhibition by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) of colony formation that was induced in normal keratinocytes. We have established cell lines from mouse papillomas and demonstrated that they are resistant to the differentiative influences of phorbol esters in culture [13]. In the present study we have analyzed 3 nontumorigenic cell lines derived from differentiation-resistant foci that developed after car-

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Abbreviations:
DMBA: 7,12-dimethylbenz[a]anthracene
DMSO: dimethylsulfoxide
MNNG: N-methyl-N′-nitro-N-nitrosoguanidine
ODC: ornithine decarboxylase
TPA: 12-*O*-tetradecanoylphorbol-13-acetate

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cinogen exposure. The effect of extracellular calcium concentration and exposure to phorbol esters on the growth and differentiation of these lines is reported here.

## MATERIALS AND METHODS

**Cell Lines** Three lines that have been previously described were used for these studies. Line F and line D were derived by treating primary newborn epidermal cells in culture with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) and 7,12-dimethylbenz[a]anthracene (DMBA), respectively [7]. Line 308 evolved from a calcium-resistant focus from adult mouse epidermis that had been exposed to DMBA in vivo [14]. All 3 lines were used between passage 9 and passage 20.

**Cell Culture Assays** Colony-forming efficiency of cell lines was measured in Eagle's medium with either 0.05 mM calcium (low calcium) or 1.2 mM calcium (high calcium) by plating 500–3000 cells per 60-mm dish in medium containing 1.2 mM calcium. After 24 h for attachment, cultures were maintained at that calcium concentration or switched to 0.05 mM calcium for study. After 14 days, cultures were fixed and stained with 0.05% crystal violet in 10% buffered formalin, and colonies with more than 10 cells were counted under a dissecting microscope. In some studies, cells were exposed to various concentrations of TPA beginning 1 day after plating. Exposure was continued throughout the course of the evaluation period, with culture medium containing fresh TPA changed 3 times weekly. The number of cornified cells was measured as described by Rice and Green [15].

Foci of calcium-resistant cells were produced by treating primary cultures of newborn BALB/c epidermal basal cells with 13.6 μM MNNG for 1 h on the 3rd day in culture as described previously [7]. At the termination of the high-calcium selection period (6 weeks after carcinogen treatment) cells were pulse-labeled with [$^3$H]thymidine (1 μCi/ml) for 1 or 12 h. Intact foci were released from the culture dish by treatment with 0.24% dispase as described [16], fixed in 10% formalin, paraffin-embedded, and sectioned. Autoradiograms were prepared from tissue sections as described [17] and were stained with hematoxylin and eosin.

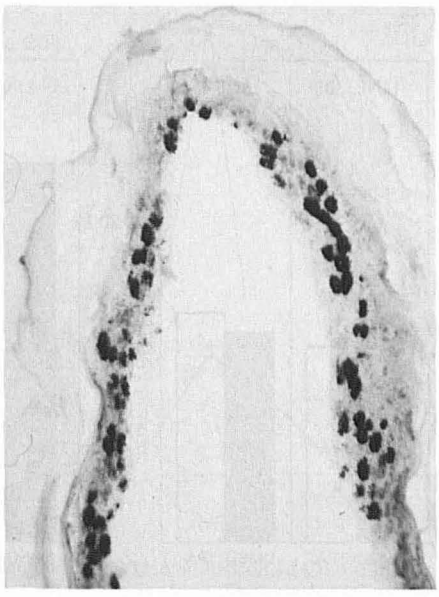

**Figure 1.** Labeling of calcium-resistant foci with [$^3$H]thymidine. Foci were produced by treatment of primary keratinocyte cultures on day 3 after plating with the carcinogen MNNG as described in [7]. Foci were labeled with [$^3$H]thymidine (1 μCi/ml) for 12 h, released intact by dispase treatment, and sectioned. Autoradiographs were prepared and sections were stained by hematoxylin and eosin. × 100.

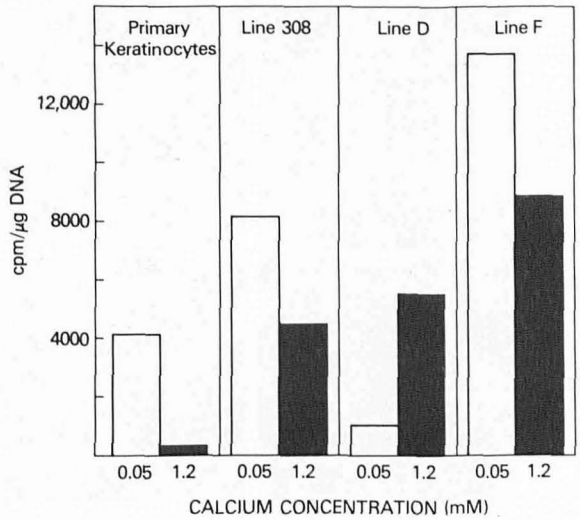


**Figure 2.** Effect of calcium switch on DNA synthesis. Primary keratinocytes or cells from lines 308, D, and F were each grown to confluence in medium with 0.05 mM calcium, then switched to medium with 1.2 mM calcium. [$^3$H]Thymidine (1 μCi/ml) was added 23 h after the calcium switch, and cells were harvested at 24 h. The specific activity of DNA (cpm/μg DNA) was determined as a measure of the rate of DNA synthesis. Values in low-calcium medium are indicated by the *open bars;* values in high-calcium are shown by the *solid bars.*

**Biochemical Assays** An estimation of the proliferation rate of cell lines under various culture conditions was performed by measuring the incorporation of [methyl-$^3$H]thymidine (New England Nuclear, Boston, Massachusetts) into DNA as described previously [18]. In some experiments, the thymidine labeling index was estimated autoradiographically [17]. Epidermal transglutaminase activity was assayed in cell lysates by incorporation of [$^3$H]putrescine (New England Nuclear) into casein as described previously [19]. Ornithine decarboxylase activity in cell lysates was quantified from the production of [$^{14}$C]$CO_2$ from [$^{14}$C]ornithine (Amersham, Arlington Heights, Illinois) as previously described [20].

**Chemicals** The tumor promoters TPA, mezerein, and 12-O-retinoylphorbol-13-acetate were obtained from LC Services, Woburn, Massachusetts.

## RESULTS

**Morphology and [$^3$H]Thymidine Labeling of Calcium-Resistant Foci** A striking characteristic of chemically induced mouse skin papillomas is the presence in autoradiographs of [$^3$H]thymidine-labeled cells in strata above the basement membrane zone [21]. This result implies that the response of initiated cells to differentiation signals has been altered; unlike normal keratinocytes, they are not obligated to cease proliferation after migration away from the basement membrane. Carcinogen-induced calcium-resistant foci were labeled in culture with [$^3$H]thymidine, released intact by dispase, and vertical cross-sections were prepared for autoradiography. Figure 1 shows the autoradiogram of a dispase-released focus, which is well organized and stratified with a pattern of differentiation similar to that seen in skin in vivo. Considerable differentiation is obvious with an extensive stratum corneum-like superficial layer. After exposure to [$^3$H]thymidine for 12 h prior to release and fixation, labeled cells are present in all the living cell layers and the labeling index is high (Fig 1). Foci labeled for only 1 h show similar suprabasal labeling (not shown). In these aspects, primary foci are similar to papillomas in mouse skin in vivo.

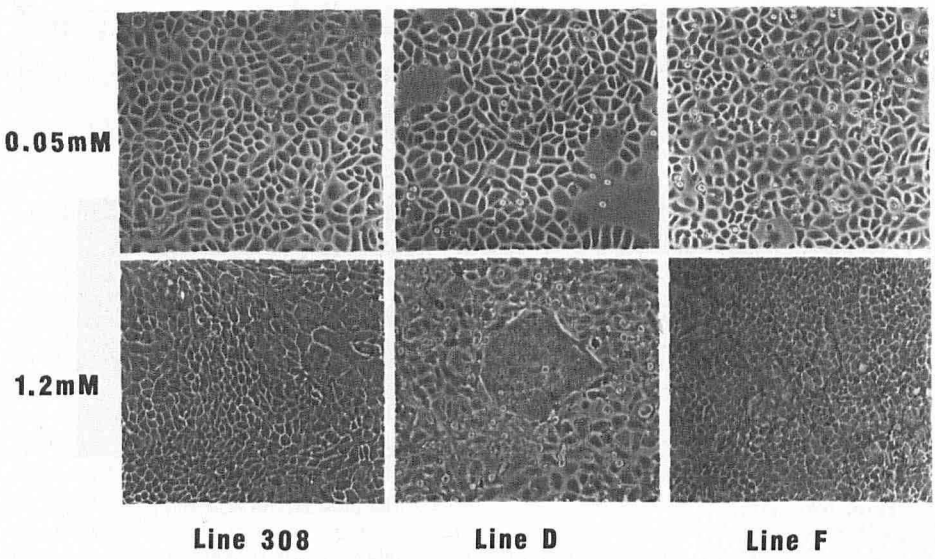


**Figure 3.** Alteration of cell morphology of lines 308, D, and F by medium calcium concentration. The cells of each line were plated in medium with either 0.05 mM or 1.2 mM calcium and grown to confluence. × 100.

**Growth Kinetics of Calcium-Resistant Cells** When cell lines were derived from foci similar to those shown in Fig 1, their growth properties were significantly altered compared to primary keratinocytes. Figure 2 demonstrates the effect of extracellular calcium levels on [$^3$H]thymidine incorporation into 3 cell lines relative to that of normal keratinocytes. Within 24 h of shifting from 0.05 mM to 1.2 mM calcium, thymidine incorporation in normal keratinocytes is inhibited by more than 90% [2]. In contrast, thymidine incorporation decreased less than 50% in lines 308 and F and increased 5-fold in line D. With prolonged growth in medium with 1.2 mM calcium, thymidine incorporation in lines 308 and D was at least as high as in medium with 0.05 mM calcium. However, line F showed diminished thymidine incorporation after long-term culture in 1.2 mM calcium, possibly related to extremely dense growth of the cells (not shown).

Growth after plating at clonal density (500–1000 cells/60-mm dish) varied considerably for the 3 cell lines. In high-calcium medium, the colony-forming efficiency of lines 308, F, and D was 18.9%, 2.0%, and 1.1%, respectively. In low-calcium medium, the colony-forming efficiency of line 308 was reduced to 4.7% with much smaller colonies than in high-calcium medium. Line D did not grow at clonal density in medium with low calcium. Line F was not tested. In contrast to these 3 lines, primary keratinocytes do not grow at clonal density in either low- or high-calcium medium.

**Failure of Calcium to Induce Differentiation Markers in Calcium-Resistant Cell Lines** All 3 cell lines grow with an altered morphology in medium with high calcium, as shown in Fig 3. However, when specific markers of differentiation were studied, calcium did not have a significant influence on their expression. For example, the percent of cornified cells attached to the culture dish (which increases from 0.06% to 0.61% in primary keratinocytes 24 h after a switch from 0.05 to 1.2 mM calcium) remained at about 0.01% in calcium-resistant cell lines switched to high-calcium medium (not shown). The activity of epidermal transglutaminase, a marker of epidermal differentiation, increases 2- to 3-fold between 24–72 h in primary keratinocytes switched from 0.05 to 1.2 mM calcium [19,22]. Transglutaminase activity was not increased in any of the calcium-resistant cell lines studied when the cells were switched from low- to high-calcium culture conditions (Table I). The high level of transglutaminase activity in line F cells was subsequently shown to be due to "tissue" transglutaminase [23] rather than epidermal transglutaminase, based on criteria of solubility and thermal stability (Lichti, unpublished observations).

**Table I.** Lack of Calcium Effect on Transglutaminase Activity in Lines 308, D, and F

| | Extracellular Calcium Concentration (cpm/mg protein/10 min × $10^{-3}$) | |
|---|---|---|
| Line | 0.05 mM | 1.20 mM |
| 308 | 2.7 ± 0.1 | 3.2 ± 0.2 |
| D | 1.4 ± 0.5 | 2.6 ± 0.8 |
| F | 194 ± 21 | 238 ± 10 |

Cells of lines 308, D, and F were grown to near confluence in medium with 1.2 mM calcium. Three days before assay, cells were either switched to medium with 0.05 mM calcium or continued in 1.2 mM calcium. Duplicate assays of transglutaminase were carried out on duplicate samples. The average values ± the range are indicated. Typical values for primary keratinocytes in low-calcium medium are 1–2 cpm/mg protein/10 min × $10^{-3}$.

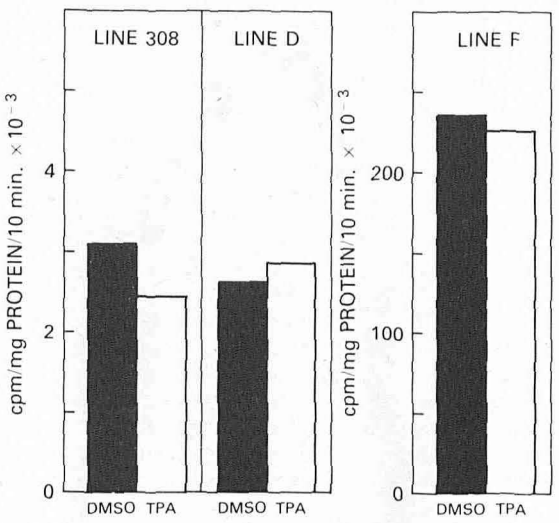


**Figure 4.** Effect of TPA on transglutaminase activity. Cells of lines 308, D, and F were grown to confluence in medium with 1.2 mM calcium. After an 8-h exposure to TPA (100 ng/ml) or DMSO solvent (0.1%), cells were harvested for transglutaminase assays. Each value represents the average of duplicate assays performed on duplicate samples, which agreed to within 10%. *Solid bars* indicate the values for DMSO-treated cultures; *open bars* indicate TPA-treated cultures.

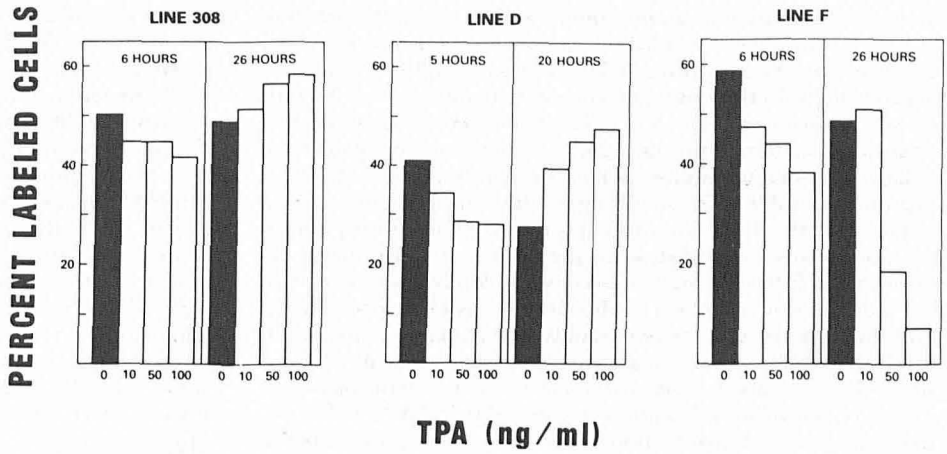


**Figure 5.** Effect of TPA on labeling index of calcium-resistant cell lines. Cells of lines 308, D, and F were grown to confluence in medium with 1.2 mM calcium. At zero time, TPA was added at a dose of 10, 50, or 100 ng/ml. DNA-synthesizing cells were labeled for 2 h with [$^3$H]thymidine and dishes were fixed at the times indicated on the figure and processed for autoradiography. Cells with 5 or more grains over the nucleus were counted as labeled. The labeling index is expressed as the percent labeled cells.

**Response of Calcium-Resistant Cells to Tumor Promoters**

Phorbol ester tumor promoters induce differentiation (as measured by cornified cells or epidermal transglutaminase activity) in a subpopulation of normal keratinocytes while stimulating proliferation in another subpopulation [11]. In either low- or high-calcium medium, TPA treatment of primary epidermal cultures induced a 5- to 12-fold increase in percentage of attached cells that are cornified at 24 h (not shown). However, after exposure of lines 308, D, and F to TPA, the number of cornified cells did not increase (not shown). In primary keratinocytes, TPA treatment also increases epidermal transglutaminase activity 2- to 5-fold in either low- or high-calcium medium. In the calcium-resistant cell lines, transglutaminase is also unchanged in response to exposure to TPA under low-calcium (not shown) or high-calcium conditions (Fig 4). Thus, these cell lines are resistant to the differentiation induced by phorbol esters as well as by increased extracellular calcium.

In primary epidermal cultures exposed to TPA (100 ng/ml) in high-calcium medium, the thymidine labeling index was reduced 60% at 21 h, after which proliferation increased. At 50 h the labeling index was 19.6% in the TPA-treated cultures compared with 9.2% in the dimethylsulfoxide (DMSO)-treated controls. The proliferative response to the tumor promoter was more rapid

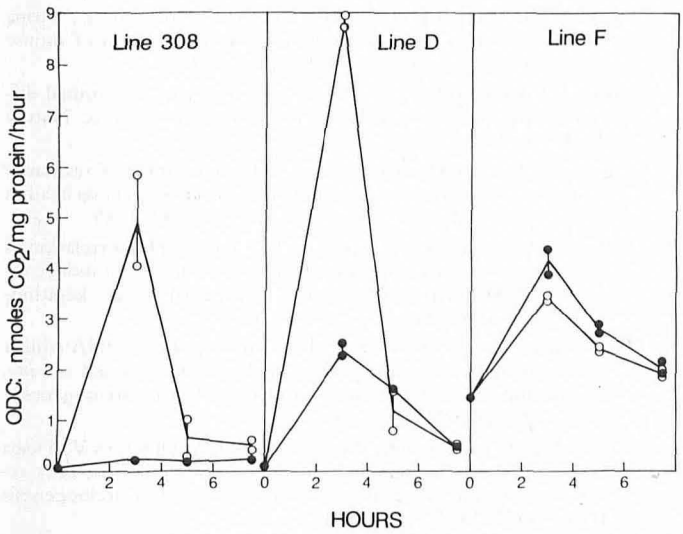


**Figure 6.** Time course of TPA induction of ODC. In this experiment, cells were plated at low density in medium with 0.05 mM calcium. When treated with TPA (100 ng/ml) or DMSO solvent, line 308 was 80% confluent, line D was about 20% confluent, and line F was 50% confluent. Cells were harvested at 3, 5, and 8 h for assay of ODC. The ODC activity is expressed as nmol $CO_2$/mg protein/h. DMSO solvent, (●—●); TPA, (○—○). In primary keratinocytes 3 h after treatment, a value of 14.7 ± 1.0 nmol $CO_2$/mg protein/h was found in TPA-treated cultures, compared with 2.4 ± 0.2 in controls.

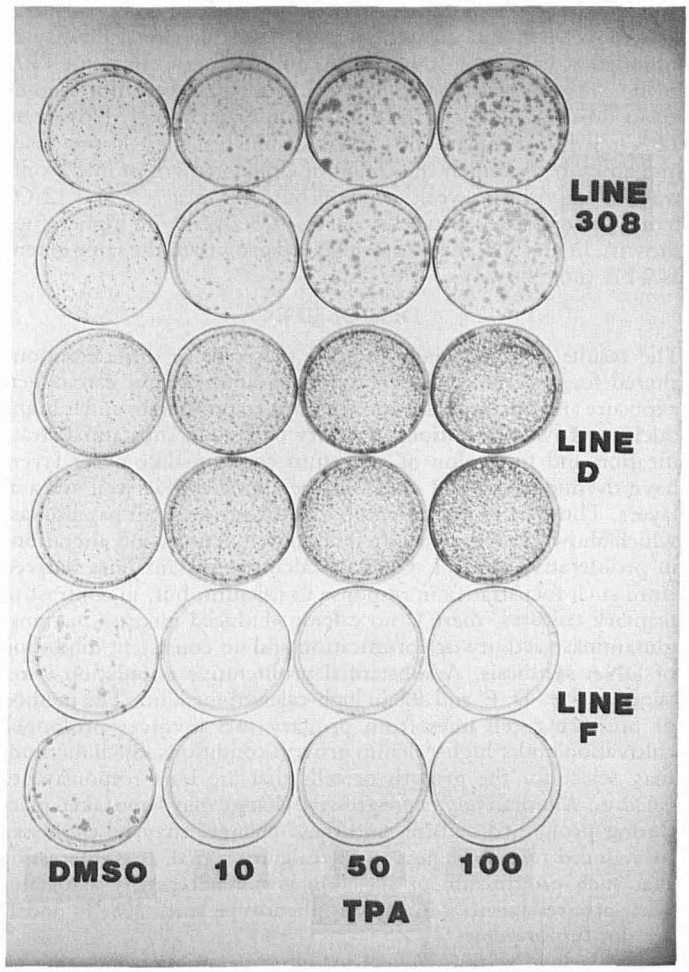


**Figure 7.** Effect of TPA on growth of cells plated at low densities. Cells of each line were plated at the following densities in medium with 1.2 mM calcium in 60-mm dishes: line 308, 1,000 cells; line D, 10,000 cells, line F, 3,000 cells. Beginning at day 1, either DMSO solvent or TPA at 10, 50, or 100 ng/ml was added to the medium. Medium was changed 3 times weekly, and dishes were stained at day 14. A comparison with primary epidermal cultures cannot be made since they do not grow when plated at clonal density.

in 2 of the cell lines, as shown in Fig 5. The thymidine labeling index of both lines 308 and D decreased at 5–6 h and increased 20–26 h after TPA treatment. This response to TPA was dose-dependent. In [$^3$H]thymidine incorporation studies (not shown), the specific activity of DNA in lines 308 and D returned to normal levels at 48 h after elevation at 24 h. In line F, the early decrease in labeling index was followed by a further decrease at 26 h after exposure to TPA at 50 or 100 ng/ml (Fig 5). The difference in response of the 3 cell lines to exposure to phorbol ester tumor promoters was confirmed when the induction of ornithine decarboxylase (ODC) activity, an enzyme closely associated with the induction of epidermal proliferation, was examined (Fig 6). Line 308 and line D were both highly responsive to induction of ODC by TPA with high activity 3 h after exposure. Line F showed no increase in ODC activity associated with the promoter exposure. In primary keratinocytes exposed to TPA, ODC activity increased 4- to 17-fold at 3–3½ h (Lichti, unpublished observations).

Long-term exposure to TPA was also associated with growth stimulation of line 308 and line D cells. Figure 7 presents the results of clonal growth assays under high-calcium growth conditions in cultures exposed to TPA from day 1 to day 14 after plating. Both the size and number of colonies are increased by TPA in a dose-dependent manner in the 2 lines. After 7 days of TPA treatment, line 308 colonies contained 49.2 ± 1.4 cells compared to 24.6 ± 1.8 cells in DMSO-treated controls. Figure 7 also shows that the growth of line F cells at clonal density is inhibited at 10, 50, or 100 ng TPA/ml. The toxic effects of TPA on line F were also apparent at 5 ng/ml, but were not seen at lower doses (0.5 or 1 ng/ml) of the tumor promoter. However, TPA failed to stimulate the growth of line F at these lower doses (not shown). A similar inhibition of clonal growth of line F cells was found with the weak tumor promoters mezerein and 12-O-retinoylphorbol-13-acetate at doses of 5 ng/ml or higher (not shown). In line 308, mezerein enhanced growth to the same extent as TPA (not shown).

## DISCUSSION

The results presented here indicate that cells in differentiation-altered foci isolated from keratinocyte cultures after carcinogen exposure and calcium selection continue to proliferate under high-calcium growth conditions. Primary foci, with substantial stratification and formation of a stratum corneum-like outer layer, have thymidine-labeled nuclei in the suprabasal as well as basal layers. Thus, these foci resemble chemically induced papillomas, which show exaggerated differentiative functions and alterations in proliferative control. Cells of calcium-resistant lines derived from such foci stratify in response to calcium, but, in contrast to primary cultures, there is no calcium-induced increase in transglutaminase activity or cornification and no consistent inhibition of DNA synthesis. A substantial proliferative population is retained in lines D, F, and 308 in high-calcium medium. The method of producing cell lines from primary foci involves prolonged cultivation under high-calcium growth conditions. Such methods may select for the growth of cells that are least responsive to calcium. Alternatively, a progressive change may have taken place during prolonged culture, and these changes may be expressed by reduced responsiveness to the calcium signal. It is interesting that such progression or selection is not necessarily associated with progression to a malignant phenotype since 308, F, and D are not tumorigenic.

The lack of responsiveness to the differentiation-inducing effects of phorbol ester tumor promoters is consistent with the proposal that such cell lines would have a growth advantage during tumor promotion. The stimulation of proliferation by tumor promoters would give cells such as those in line 308 and line D an additional selective advantage. In other studies, lines D and 308 were shown to be resistant to the cytotoxic effects of benzoyl peroxide, another tumor promoter for mouse skin [24]. If these cells represent initiated cells, then the resistant phenotype would give them a growth advantage in epidermis exposed to the cytotoxic concentrations of benzoyl peroxide required for promotion [25].

The underlying difference that distinguishes these cell lines from normal cells in their response to TPA is not clear. Differential responses to tumor promoters among normal and neoplastic cells have been demonstrated for several other model systems. Transformed human keratinocyte cell lines are resistant to the cytotoxic effects of phorbol esters in culture [26] and neoplastic human bronchial epithelial cells are resistant to the differentiation-inducing effects of phorbol esters, unlike their normal counterparts [27]. Preneoplastic colonic epithelial cells are selectively stimulated to proliferate by tumor promoters, while normal colonic epithelial cells do not respond [28]. It has been known for some time that preneoplastic liver cells are resistant to the cytotoxic effects of certain carcinogens that are used as tumor promoters during liver carcinogenesis [29]. We have recently isolated cells from chemically induced papillomas and derived cell lines in culture [13]. Cells of these lines are all responsive to the growth-promoting effects of phorbol ester tumor promoters and are resistant to their differentiation-inducing effects. In this regard they are similar to the initiated cell lines that are described here. Thus, these differential responses of normal and neoplastic cells to tumor-promoting stimuli may be a general phenomenon among tissues in which promotion is a required step in the carcinogenesis process. While the nature of the differential response may be tissue-specific (e.g., differentiation-related, cytotoxicity-related, or proliferation-related), the selective advantage afforded to the neoplastic cell may be necessary for clonal expansion during the process of promotion.

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